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JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of modulating endothelial cell activity"

The invention is described in the following statement:

A METHOD OF MODULATING ENDOTHELIAL CELL ACTIVITY

FIELD OF THE INVENTION

5 The present invention relates generally to a method of modulating endothelial cell functional characteristics relative to normal endothelial cell functional characteristics and to agents useful for same. More particularly, the present invention relates to a method of modulating vascular endothelial cell proliferation and differentiation characteristics relative to that of normal endothelial cells by modulating the functional levels of

10 intracellular sphingosine kinase. The method of the present invention is useful, *inter alia*, in relation to the treatment and/or prophylaxis of conditions which are characterised by inadequate endothelial cell functioning and may include conditions such as vascular engraftment, organ transplantation or wound healing. Further, the method of the present invention facilitates the development of agents, such as functionally manipulated

15 endothelial cell populations, for a range of therapeutic and/or prophylactic uses.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are

20 collected alphabetically at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that the prior art forms part of the common general knowledge in Australia.

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The survival and proliferation of cells is dependent upon an adequate supply of oxygen and nutrients and the removal of toxins. Angiogenesis is the name given to the development of new capillaries from pre-existing blood vessels. In order for stimulated endothelial cells to form a new blood vessel, they must proliferate, migrate and invade the surrounding tissue.

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In adult mammals, the vasculature is quiescent, except during the physiological cycle of reproduction or in the case of wound healing. Further, additional requirements in terms of oxygen or nutrients will usually result in sprouting of new capillaries from pre-existing vessels. Local hyper-vascularisation is thought to result from release by tissues of soluble media which has induced the switch of the quiescent endothelial cell phenotype to the activated one, in order for endothelial cells to be able to respond to mitogenic signals. The release of mitogenic growth factors allows the activation of the receptors that signal for cell migration, proliferation and differentiation into new capillaries and thereby switches the activated phenotype to an angiogenic phenotype.

There is an ongoing need to develop methods for facilitating angiogenesis, such as in the context of vascularisation of grafts or wound healing. In terms of working with and manipulating endothelial cells, there are certain inherent functional limitations such as the requirement for attachment and cell spreading mediated anti-apoptotic signals in order to maintain endothelial cell viability. Further, activation of endothelial cell differentiation generally results in loss of the haematopoietic cell marker CD34. This irreversibly alters the phenotype of the activated endothelial cells.

In light of the significant interest in promoting angiogenesis in both the *in vitro* and *in vivo* environments, there is a need to develop means of both facilitating the maintenance of optimal endothelial cell phenotypes and promoting optimal endothelial cell growth. In work leading up to the present invention, it has been determined that over expression of the human sphingosine kinase gene in human endothelial cells results in enhanced endothelial cell proliferation and cell survival relative to normal cells. Further, sphingosine kinase over expression has been determined to maintain the endothelial cell haematopoietic phenotype, as characterised by the expression of CD34, despite the induction of endothelial cell proliferation. Accordingly, there is now provided a means of facilitating the therapeutic manipulation of endothelial cell proliferation and differentiation based on modulation of intracellular sphingosine kinase levels.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising",
5 will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention is directed to a method of modulating one or more endothelial cell functional characteristics, said method comprising modulating the
10 functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell activity.

In another aspect there is provided a method of modulating one or more vascular
15 endothelial cell functional characteristics, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said vascular endothelial cell relative to normal vascular endothelial cell characteristics.

20 In yet another aspect there is provided the method of modulating one or more CD34⁺ endothelial cell functional characteristics, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said CD34⁺ endothelial cell relative to normal CD34⁺ endothelial cell characteristics.

25 In still another aspect there is provided a method of modulating vascular endothelial cell proliferation, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level enhances the proliferation of said endothelial cell relative to normal endothelial cell proliferation.

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In still yet another aspect there is provided a method of modulating vascular endothelial viability, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level enhances the viability of said vascular endothelial cell relative to normal endothelial cell viability.

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In yet still another aspect there is provided a method of modulating the CD34⁺ endothelial cell progenitor phenotype, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level maintains the CD34⁺ endothelial cell progenitor phenotype.

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A further aspect of the present invention is directed to a method of modulating one or more endothelial cell functional characteristics in a mammal, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell characteristics.

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In another further aspect said method is directed to modulating one or more vascular endothelial cell functional characteristics in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell characteristics.

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In yet another further aspect there is provide a method of modulating vascular endothelial cell proliferation in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level enhances the proliferation of said endothelial cell relative to normal endothelial cell proliferation.

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In still another further aspect there is provided a method of modulating vascular endothelial cell viability in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said

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sphingosine kinase level enhances the viability of said vascular endothelial cell relative to normal endothelial cell viability.

In yet another aspect there is provided a method of modulating the CD34⁺ endothelial cell progenitor phenotype in a mammal, said method comprising modulating the functional level of said sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level maintains the CD34⁺ endothelial cell progenitor phenotype.

Another aspect of the present invention contemplates a method for the treatment and/or prophylaxis of a condition characterised by inadequate endothelial cell functioning in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

Yet another aspect of the present invention provides a method for the treatment and/or prophylaxis of a condition characterised by inadequate vascular endothelial cell functioning in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

In still another aspect there is provided a method for the treatment and/or prophylaxis of a condition characterised by inadequate vascular endothelial cell functioning in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce over-expression of a functional level of sphingosine kinase.

Another aspect of the present invention relates to the use of an agent capable of modulating the functional level of sphingosine kinase in the manufacture of a medicament for the modulation of one or more endothelial cell functional characteristics in a mammal wherein

inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

- 5 In another aspect, the present invention relates to the use of sphingosine kinase or a nucleic acid encoding sphingosine kinase in the manufacture of a medicament for the modulation of one or more endothelial cell functional characteristics in a mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cells relative to normal endothelial cell functional
 10 characteristics.

- In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. Still another aspect of the present
 15 invention is directed to a method of generating an endothelial cell, which endothelial cell is characterised by the modulation of one or more functional characteristics relative to normal endothelial cell functional characteristics, said method comprising inducing over-expression of the functional level of sphingosine kinase in said cell.

- 20 Yet another aspect of the present invention is directed to the endothelial cells which are generated in accordance with the methods defined herein.

- Still yet another aspect of the present invention is directed to the use of endothelial cells developed in accordance with the method defined herein in the treatment and/or
 25 prophylaxis of conditions characterised by inadequate endothelial cell functioning.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the over-expression of sphingosine kinase in endothelial cells is achieved by retrovirus or adenovirus mediated gene transfer. For retrovirus, infected cells are selected with the antibiotic G418. Clones of selected cells are pooled and expanded. This method gives long term endothelial cells lines. For adenovirus, 100% of the cells are infected (as judged by the green fluorescence protein marker) and transiently express the sphingosine kinase gene.

- Transfected cells were assessed for endothelial cells functions such as cell survival, proliferation and antigen expression.

Figure 2 is a graphical representation of the mean relative sphingosine kinase activity of endothelial cells over-expressing sphingosine kinase gene compared to empty vector infected control cells when delivered via retrovirus. This is the mean \pm SEM of sphingosine kinase activity of 5 individual endothelial cells lines.

Sphingosine kinase activity is assessed by $\gamma^{32}\text{P}$ ATP incorporation into sphingosine to generate sphingosine-1-phosphate with resolution by TLC.

Figure 3 is a graphical representation of the over-expression of sphingosine kinase resulting in an increase in the ability of cells to proliferate in complete medium. Proliferation is measured using the commercially available MTS assay and is measured at OD 490 nm. The rate of proliferation over a 3 day period is given.

Over-expression of sphingosine kinase results in the ability of the endothelial cells to survive when plated under serum-free conditions. Under such serum-free conditions, normal endothelial cells either fail to proliferate or apoptose. Survival is measured by the MTS assay over a 3 day period.

Figure 4 is a graphical representation of the over-expression of sphingosine kinase resulting in inhibition of apoptosis. Cells were plated either with normal complete endothelial cells growth medium or in serum-free medium. Caspase 3 activity was measured using a commercially available kit and is based on the caspase 3 dependent conversion of substrate and generation of fluorophor.

Caspase 3 is a central enzyme involved in the apoptotic pathway. A decrease in caspase 3 activity indicates a decrease in number of apoptotic cells.

Figure 5 is a graphical representation of the fact that the sphingosine kinase mediated increase in cell survival is mediated through the PI3 Kinase pathway. This was assessed by the use of the PI3 Kinase inhibitor LY264002 which inhibits the sphingosine kinase induced cell survival (as measured by the MTS assay).

Figure 6 is a graphical representation of the over-expression of sphingosine kinase promoting cell survival of endothelial cells grown in suspension.

Cells were plated in non tissue culture treated microtitre wells for 24 hours. This inhibits matrix attachment and cell spreading. Failure to engage integrin receptors involved in matrix attachment and inhibition of cell spreading results in apoptotic cell death, as shown in vector control (EV). Survival was measured by the MTS assay. Adenovirus was used for mediating gene transfer.

Figure 7 is a graphical representation of the over-expression of sphingosine kinase in endothelial cells resulting in maintenance of CD34 antigen expression. CD34 antigen is highly expressed on haematopoietic progenitor cells and on quiescent endothelial cells. It is rapidly lost on proliferating endothelial cells or on activation by inflammatory cytokines (eg TNF). Thus, although these sphingosine kinase over-expressing cells are more rapidly proliferating than control cells, levels of CD34 are being maintained. Antigen expression is assessed by flow cytometry using commercially available anti-CD34 antibodies. Results

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are expressed as the mean fluorescence intensity of three separate experiments. Adenovirus was used for mediated gene transfer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the determination that endothelial cell functional characteristics can be modulated, relative to that of normal endothelial cells, by over expressing sphingosine kinase. Specifically, it has been determined that over expressing sphingosine kinase facilitates enhanced cell proliferation and cell survival in the absence of normal anti apoptotic signals. Further, to the extent that the method of the present invention is applied to CD34 expressing endothelial cells, their progenitor-like properties can be maintained despite the onset of proliferation. Accordingly, the method of the present invention now permits the rational design of therapeutic and/or prophylactic methods for treating conditions characterised by inadequate endothelial cell functioning or for otherwise facilitating endothelial expansion either *in vitro* or *in vivo*. The determinations detailed herein also facilitate the development of both cellular and non-cellular agents for use in the context of treating the conditions detailed above or otherwise seeding and/or expanding an endothelial cell population.

Accordingly, one aspect of the present invention is directed to a method of modulating one or more endothelial cell functional characteristics, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell functional characteristics.

Reference to "endothelial cell" should be understood as a reference to the squamous epithelial cells which line the blood vessels, lymphatics or other serous cavities such as fluid filled cavities. Unlike most epithelial cells, endothelial cells are mesodermally derived. The phrase "endothelial cells" should also be understood as a reference to cells which exhibit one or more of the morphology, phenotype and/or functional activity of endothelial cells and is also a reference to mutants or variants thereof. "Variants" include, but are not limited to, cells exhibiting some but not all of the morphological or phenotypic features or functional activities of endothelial cells at any differentiative stage of development. "Mutants" include, but are not limited to, endothelial cells which have been

naturally or non-naturally modified such as cells which are genetically modified.

It should also be understood that the endothelial cells of the present invention may be at any differentiative stage of development. Accordingly, the cells may be immature and therefore functionally incompetent in the absence of further differentiation, such as CD34⁺ progenitor cells. In this regard, highly immature cells such as stem cells, which retain the capacity to differentiate into endothelial cells, should nevertheless be understood to satisfy the definition of "endothelial cell" as utilised herein due to their *capacity* to differentiate into endothelial cells under appropriate conditions. Preferably, the subject endothelial cell is a vascular endothelial cell and even more preferably a CD34⁺ endothelial cell.

Accordingly, there is more particularly provided a method of modulating one or more vascular endothelial cell functional characteristics, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said vascular endothelial cell relative to normal vascular endothelial cell characteristics.

Still more particularly, there is provided the method of modulating one or more CD34⁺ endothelial cell functional characteristics, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said CD34⁺ endothelial cell relative to normal CD34⁺ endothelial cell characteristics.

Reference to endothelial cell "functional characteristics" should be understood as reference to any one or more of the functional characteristics which an endothelial cell is capable of exhibiting. This includes, for example, proliferation, differentiation, migration, maintenance of viability in either a quiescent or active state and cell surface molecule expression. In the context of the present invention, it has been determined that over-expression of intracellular sphingosine kinase can induce modulation of one or more endothelial cell functional characteristics relative to normal endothelial cell functional characteristics. By "normal" is meant the characteristic or range of characteristics which

are exhibited by cells expressing physiologically normal levels of sphingosine kinase. In this regard, it should be understood that physiologically normal levels of sphingosine kinase will equate to a range of levels depending on whether a given endothelial cell is in a quiescent or activated state. Accordingly, the range of functional characteristics which an endothelial cell can perform will be usually defined by the state of differentiation of the endothelial cell and the level of expression of sphingosine kinase.

Without limiting the present invention to any one theory or mode of action, where physiologically normal levels of sphingosine kinase are expressed, a vascular endothelial cell may exhibit one or more characteristics including, but not limited to:

- the maintenance of a viable but quiescent state
- the capacity to differentiate under appropriate stimulatory conditions (for example, maturation from CD34⁺ progenitor state to a more mature endothelial cell phenotype)
- the capacity to proliferate
- the maintenance of viability in an activated state
- the capacity to modulate cell surface molecule expression (for example, as an indicator of maturation or activation state).

It should be understood, however, that under normal physiological conditions there are certain inherent functional limitations to which endothelial cells are subject. For example, in order to maintain viability, vascular endothelial cells require exposure to certain anti-apoptotic signals such as those which are generated as a result of normal vascular endothelial cell attachment and cell spreading. Accordingly, in the absence of such signals – as may occur where cells are grown *in vitro* in suspension – unwanted apoptosis will occur. In another example, whereas immature, quiescent endothelial cells express the

cell surface haematopoietic marker CD34, the stimulation and induction of endothelial cell proliferation (for example, in order to facilitate angiogenesis) results in loss of CD34 expression and, by definition, the development of an irreversible and more mature phenotype. In certain circumstances, such as where one is seeking to expand the CD34⁺ endothelial cell population, this can prove to be a disadvantage since the signals which initiate proliferation also lead to phenotypic maturation.

As detailed hereinbefore, it has been determined that over-expressing sphingosine kinase in an endothelial cell results in the induction of functional characteristics which are not generally observed when sphingosine kinase is expressed in the normal range. Accordingly, reference to "modulating" the functional characteristics of an endothelial cell "relative to" normal endothelial cell characteristics should be understood to mean that the over-expression of sphingosine kinase levels results in the induction of one or more characteristics which are not generally observed in the context of cells expressing sphingosine kinase in the normal range. It should be understood, however, that the subject characteristics may replace entirely the range of normal functional characteristics of an endothelial cell or one or more of these characteristics may be expressed together with one or more normal characteristics. Without limiting the present invention in any way, examples of characteristics which may be induced in endothelial cells over-expressing sphingosine kinase levels include, but are not limited to:

- improved proliferative characteristics both in terms of an increased rate/extent of proliferation and the requirement for only minimal environmental/cell culture conditions under which proliferation can occur (herein referred to as "enhanced proliferation")
- Improved cell viability. This may occur either at the level of down regulating apoptosis or preventing otherwise induced cell death. For example, cell survival under conditions of stress (such as the removal of tissue culture supplements in the *in vitro* environment) is facilitated as is the down regulation of apoptosis which would normally occur in the absence of

the anti-apoptotic signals which are provided as a result of integrin receptor engagement during matrix attachment and cell spreading. This is particularly relevant, for example, where *in vitro* cell culture populations are required to be maintained in suspension (herein referred to as "enhanced viability").

changed differentiation pathways. In particular, whereas the CD34 haematopoietic cell surface marker is down regulated upon stimulation of endothelial cell progenitor proliferation or the proliferation of quiescent CD34⁺ endothelial cells, over-expression of sphingosine kinase results in maintenance of both CD34 expression and the progenitor phenotype of these cells despite the onset of proliferation/expansion (herein referred to as "maintaining the CD34⁺ endothelial cell progenitor phenotype").

The subject functional characteristic modulation is therefore preferably:

- (i) enhanced proliferation;
- (ii) enhanced viability; and/or
- (iii) maintaining the CD34⁺ endothelial cell progenitor phenotype.

Accordingly, in one preferred embodiment there is provided a method of modulating vascular endothelial cell proliferation, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level enhances the proliferation of said endothelial cell relative to normal endothelial cell proliferation.

In another embodiment there is provided a method of modulating vascular endothelial viability, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level enhances the viability of said vascular endothelial cell relative to normal endothelial cell viability.

In yet another preferred embodiment there is provided a method of modulating the CD34⁺ endothelial cell progenitor phenotype, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level maintains the CD34⁺ endothelial cell progenitor phenotype.

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In accordance with these preferred embodiments, most preferably said modulation is up regulation of the subject functional characteristic.

Reference to "sphingosine kinase" should be understood as reference to all forms of this protein and to functional derivatives, homologues, analogues, chemical equivalents or mimetics thereof. This includes, for example, any isoforms which arise from alternative splicing of the subject sphingosine kinase mRNA or functional mutants or polymorphic variants of these proteins.

10 As detailed hereinbefore, it has been determined that inducing levels of intracellular sphingosine kinase which are higher than those which can be expressed by a normal endothelial cell results in the induction of unique functional characteristics. Accordingly, reference to "functional level" of sphingosine kinase should be understood as a reference to the level of sphingosine kinase *activity* which is present in any given cell as opposed to the concentration of sphingosine kinase, *per se*. Although an increase in the intracellular concentration of sphingosine kinase will generally correlate to an increase in the level of sphingosine kinase functional activity which is observed in a cell, the person skilled in the art would also understand that increases in the level of activity can be achieved by means other than merely increasing absolute intracellular sphingosine kinase concentrations. For example, one might utilise forms of sphingosine kinase which exhibit an increased half life or otherwise exhibit enhanced activity. Reference to "over-expressing" the subject sphingosine kinase level should therefore be understood as a reference to up regulating intracellular sphingosine kinase to a *functional* level which is greater than that expressed under the normal physiological conditions for a given endothelial cell or to the up-regulation of sphingosine kinase levels to any level of functionality but where that up-regulation event is one which is artificially effected rather than being an increase which

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has occurred in the subject cell due to the effects of naturally occurring physiology. It should be understood, however, that the means by which up-regulation is achieved may be artificial means which seek to mimic a physiological pathway – for example introducing a hormone or other stimulatory molecule. Accordingly, the term "expressing" is not
5 intended to be limited to the notion of sphingosine kinase gene transcription and translation. Rather, and as discussed in more detail hereinafter, it is a reference to an outcome, being the establishment of a higher functional level of sphingosine kinase than is found under normal physiological conditions in an endothelial cell at a particular point in time (ie. as detailed hereinbefore, it includes non-naturally occurring increases in
10 sphingosine kinase level, even where those increases may fall within the normal physiological range which one might observe).

Reference to "modulating" in the context of endothelial cell functional characteristics should be understood as a reference to inducing functional characteristics which are not
15 normally observed in the subject endothelial cell. This has been described in significant detail hereinbefore. In the context of the functional level of sphingosine kinase, reference to "modulating" should be understood as a reference to up regulating or down regulating the functional level of sphingosine kinase. Determining the specific optimum level to which the sphingosine kinase should be up or down-regulated in order to achieve the
20 desired phenotypic change for any given endothelial cell type is a matter of routine procedure. The person of skill in the art would be familiar with methods of determining such a level.

The present invention is preferably directed to up regulating the functional level of
25 sphingosine kinase as a means of introducing unique functional characteristics to a population of endothelial cells. However, it should nevertheless be understood that there may be circumstances in which it is desirable to down regulate the functional level of sphingosine kinase in order to obviate the expression of these characteristics. For example, one may seek to up regulate the functional level of sphingosine kinase in the context of a
30 defined population of endothelial cells for a period of time sufficient to achieve a particular objective. However, once that objective has been achieved one would likely seek to down

regulate the intracellular functional level of sphingosine kinase, to the extent that it is not transient, such that it is no longer over-expressed and the subject endothelial cells thereby take on a normal phenotype. In another example, one may identify certain disease conditions which are in fact characterised by an over-expression of the functional level of sphingosine kinase, for example due to the impact of genetic mutations. In such a situation, one may observe uncontrolled endothelial cell proliferation which could lead to tumour formation. Where such a situation exists, one may seek to down regulate the functional level of sphingosine kinase as a means of restoring a normal phenotypic profile to the endothelial cells in issue. Accordingly, the present invention, although preferably directed to up regulating the sphingosine kinase functional level in order to introduce unique phenotypic properties to the population of endothelial cells, should nevertheless be understood to also extend to down regulation of a naturally or non-naturally induced state of sphingosine kinase over-expression.

As detailed above, reference to "modulating" sphingosine kinase functional levels is a reference to either up regulating or down regulating these levels. Such modulation may be achieved by any suitable means and includes:

- (i) Modulating absolute levels of the active or inactive forms of sphingosine kinase (for example increasing or decreasing intracellular sphingosine kinase concentrations) such that either more or less sphingosine kinase is available for activation and/or to interact with its downstream targets.
- (ii) Agonising or antagonising sphingosine kinase such that the functional effectiveness of any given sphingosine kinase molecule is either increased or decreased. For example, increasing the half life of sphingosine kinase may achieve an increase in the overall level of sphingosine kinase activity without actually necessitating an increase in the absolute intracellular concentration of sphingosine kinase. Similarly, the partial antagonism of sphingosine kinase, for example by coupling sphingosine kinase to a molecule that introduces some steric hindrance in relation to the binding of sphingosine kinase to its downstream targets, may act to reduce,

although not necessarily eliminate, the effectiveness of sphingosine kinase signalling. Accordingly, this may provide a means of down-regulating sphingosine kinase functioning without necessarily down-regulating absolute concentrations of sphingosine kinase.

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In terms of achieving the up or down-regulation of sphingosine kinase functioning, means for achieving this objective would be well known to the person of skill in the art and include, but are not limited to:

- 10 (i) Introducing into a cell a nucleic acid molecule encoding sphingosine kinase or functional equivalent, derivative or analogue thereof in order to up-regulate the capacity of said cell to express sphingosine kinase.
- 15 (ii) Introducing into a cell a proteinaceous or non-proteinaceous molecule which modulates transcriptional and/or translational regulation of a gene, wherein this gene may be a sphingosine kinase gene or functional portion thereof or some other gene which directly or indirectly modulates the expression of the sphingosine kinase gene.
- 20 (iii) Introducing into a cell the sphingosine kinase expression product (in either active or inactive form) or a functional derivative, homologue, analogue, equivalent or mimetic thereof.
- 25 (iv) Introducing a proteinaceous or non-proteinaceous molecule which functions as an antagonist to the sphingosine kinase expression product.
- (v) Introducing a proteinaceous or non-proteinaceous molecule which functions as an agonist of the sphingosine kinase expression product.
- 30 The proteinaceous molecules described above may be derived from any suitable source such as natural, recombinant or synthetic sources and includes fusion proteins or molecules

which have been identified following, for example, natural product screening. The reference to non-proteinaceous molecules may be, for example, a reference to a nucleic acid molecule or it may be a molecule derived from natural sources, such as for example natural product screening, or may be a chemically synthesised molecule. The present invention contemplates analogues of the sphingosine kinase expression product or small molecules capable of acting as agonists or antagonists. Chemical agonists may not necessarily be derived from the sphingosine kinase expression product but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to meet certain physiochemical properties. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing sphingosine kinase from carrying out its normal biological function, such as molecules which prevent its activation or else prevent the downstream functioning of activated sphingosine kinase. Antagonists include monoclonal antibodies and antisense nucleic acids which prevent transcription or translation of sphingosine kinase genes or mRNA in mammalian cells. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNazymes, RNA aptamers, antibodies or molecules suitable for use in cosuppression. The proteinaceous and non-proteinaceous molecules referred to in points (i)-(v), above, are herein collectively referred to as "modulatory agents".

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising the sphingosine kinase gene or functional equivalent or derivative thereof with an agent and screening for the modulation of sphingosine kinase protein production or functional activity, modulation of the expression of a nucleic acid molecule encoding sphingosine kinase or modulation of the activity or expression of a downstream sphingosine kinase cellular target. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the readout of reporters of sphingosine kinase activity such as luciferases, CAT and the like.

It should be understood that the sphingosine kinase gene or functional equivalent or derivative thereof may be naturally occurring in the cell which is the subject of testing or it

may have been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, inter alia, screening for agents which down regulate sphingosine kinase activity, at either the nucleic acid or expression product levels, or the gene may require activation - thereby providing a model useful for, inter alia, screening for agents which up regulate sphingosine kinase expression. Further, to the extent that a sphingosine kinase nucleic acid molecule is transfected into a cell, that molecule may comprise the entire sphingosine kinase gene or it may merely comprise a portion of the gene such as the portion which regulates expression of the sphingosine kinase product. For example, the sphingosine kinase promoter region may be transfected into the cell which is the subject of testing. In this regard, where only the promoter is utilised, detecting modulation of the activity of the promoter can be achieved, for example, by ligating the promoter to a reporter gene. For example, the promoter may be ligated to luciferase or a CAT reporter, the modulation of expression of which gene can be detected via modulation of fluorescence intensity or CAT reporter activity, respectively.

In another example, the subject of detection could be a downstream sphingosine kinase regulatory target, rather than sphingosine kinase itself. Yet another example includes sphingosine kinase binding sites ligated to a minimal reporter. For example, modulation of sphingosine kinase activity can be detected by screening for the modulation of the functional activity in an endothelial cell. This is an example of an indirect system where modulation of sphingosine kinase expression, *per se*, is not the subject of detection. Rather, modulation of the molecules which sphingosine kinase regulates the expression of, are monitored.

25 These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as the proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries. These methods will also facilitate the detection of agents which bind either the sphingosine kinase nucleic acid molecule or
30 expression product itself or which modulate the expression of an upstream molecule, which upstream molecule subsequently modulates sphingosine kinase expression or

expression product activity. Accordingly, these methods provide a mechanism for detecting agents which either directly or indirectly modulate sphingosine kinase expression and/or activity.

5 The agents which are utilised in accordance with the method of the present invention may take any suitable form. For example, proteinaceous agents may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules used, linked, bound or otherwise associated with the proteins such as amino acids, lipid, carbohydrates or other peptides, polypeptides or
10 proteins. Similarly, the subject non-proteinaceous molecules may also take any suitable form. Both the proteinaceous and non-proteinaceous agents herein described may be linked, bound otherwise associated with any other proteinaceous or non-proteinaceous molecules. For example, in one embodiment of the present invention, said agent is associated with a molecule which permits its targeting to a localised region.

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The subject proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of sphingosine kinase or the activity of the sphingosine kinase expression product. Said molecule acts directly if it associates with the sphingosine kinase nucleic acid molecule or expression product to modulate expression or
20 activity, respectively. Said molecule acts indirectly if it associates with a molecule other than the sphingosine kinase nucleic acid molecule or expression product which other molecule either directly or indirectly modulates the expression or activity of the sphingosine kinase nucleic acid molecule or expression product, respectively. Accordingly, the method of the present invention encompasses the regulation of
25 sphingosine kinase nucleic acid molecule expression or expression product activity via the induction of a cascade of regulatory steps.

The term "expression" in this context refers to the transcription and translation of a nucleic acid molecule. Reference to "expression product" is a reference to the product produced
30 from the transcription and translation of a nucleic acid molecule.

"Derivatives" of the molecules herein described (for example sphingosine kinase or other proteinaceous or non-proteinaceous agents) include fragments, parts, portions or variants from either natural or non-natural sources. Non-natural sources include, for example, recombinant or synthetic sources. By "recombinant sources" is meant that the cellular source from which the subject molecule is harvested has been genetically altered. This may occur, for example, in order to increase or otherwise enhance the rate and volume of production by that particular cellular source. Parts or fragments include, for example, active regions of the molecule. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in a sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins, as detailed above.

Derivatives also include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, sphingosine kinase or derivative thereof may be fused to a molecule to facilitate its entry into a cell. Analogues of the molecules contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

Derivatives of nucleic acid sequences which may be utilised in accordance with the method of the present invention may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules utilised in the present invention

include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

- 5 A "variant" of sphingosine kinase should be understood to mean molecules which exhibit at least some of the functional activity of the form of sphingosine kinase of which it is a variant. A variation may take any form and may be naturally or non-naturally occurring. A mutant molecule is one which exhibits modified functional activity.
- 10 A "homologue" is meant that the molecule is derived from a species other than that which is being treated in accordance with the method of the present invention. This may occur, for example, where it is determined that a species other than that which is being treated produces a form of sphingosine kinase which exhibits similar and suitable functional characteristics to that of the sphingosine kinase which is naturally produced by the subject
- 15 undergoing treatment.

- Chemical and functional equivalents should be understood as molecules exhibiting any one or more of the functional activities of the subject molecule, which functional equivalents may be derived from any source such as being chemically synthesised or identified via
- 20 screening processes such as natural product screening. For example chemical or functional equivalents can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening.
- 25 For example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (eg., Bunin BA, *et al.* (1994) *Proc. Natl. Acad. Sci. USA*, 91:4708-4712; DeWitt SH, *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, 90:6909-6913). Briefly, at each successive synthetic step, one of a plurality of
- 30 different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the

different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing sphingosine kinase analogues which exhibit properties such as more potent pharmacological effects. Sphingosine kinase or a functional part thereof may according to the present invention be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

In addition to screening for molecules which mimic the activity of sphingosine kinase, it may also be desirable to identify and utilise molecules which function agonistically or antagonistically to sphingosine kinase in order to up or down-regulate the functional activity of sphingosine kinase in relation to modulating endothelial cell growth. The use of such molecules is described in more detail below. To the extent that the subject molecule is proteinaceous, it may be derived, for example, from natural or recombinant sources including fusion proteins or following, for example, the screening methods described above. The non-proteinaceous molecule may be, for example, a chemical or synthetic molecule which has also been identified or generated in accordance with the methodology identified above. Accordingly, the present invention contemplates the use of chemical analogues of sphingosine kinase capable of acting as agonists or antagonists. Chemical agonists may not necessarily be derived from sphingosine kinase but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of sphingosine kinase. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing sphingosine kinase from carrying out its normal biological functions. Antagonists include monoclonal antibodies specific for sphingosine kinase or parts of sphingosine kinase.

Analogues of sphingosine kinase or of sphingosine kinase agonistic or antagonistic agents contemplated herein include, but are not limited to, modifications to side chains, incorporating unnatural amino acids and/or derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the analogues. The specific form which such modifications can take will depend on whether the subject molecule is proteinaceous or non-proteinaceous. The nature and/or suitability of a particular modification can be routinely determined by the person of skill in the art.

For example, examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate;

trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH_4 .

- 5 The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea

- 10 formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

- 15
- 20 Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

- 25 Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

- 30 Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline,

phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated herein is shown in Table 1.

TABLE 1

	Non-conventional amino acid	Code	Non-conventional amino acid	Code
5	α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
	aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
			L-N-methylaspartic acid	Nmasp
10	aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
	aminonorbomyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
			L-N-methylglutamic acid	Nmglu
	cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
	cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15	D-alanine	Dal	L-N-methylleucine	Nmleu
	D-arginine	Darg	L-N-methyllysine	Nmlys
	D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
	D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
	D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
	D-lysine	Dlys	L-N-methylthreonine	Nmthr
25	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg
	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib

	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgab
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpn
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
5	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Nom
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
10	D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
15	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Nedec
20	D- α -methylvaline	Dmval	N-cyclododecylglycine	Nedod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Nepro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
25	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmglu	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
30	D-N-methylleucine	Dnmleu	N-(3-indolylethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchcxa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn

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	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
5	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
10	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
15	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
20	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
25	N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
	carbonylmethyl)glycine		carbonylmethyl)glycine	
	1-carboxy-1-(2,2'-diphenyl-Nmbc			
	ethylamino)cyclopropane			

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with $n=1$ to $n=6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

The method of the present invention contemplates the modulation of endothelial cell functioning both *in vitro* and *in vivo*. Although the preferred method is to treat an individual *in vivo*, it should nevertheless be understood that it may be desirable that the method of the invention be applied in an *in vitro* environment. For example, one may seek to initiate angiogenesis by inducing endothelial cell proliferation in accordance with the method of the present invention in a donor graft prior to its introduction to a host. In another example, one may seek to expand populations of endothelial cells in culture prior to their localised introduction to a subject who is undergoing treatment. In yet another example, the method of the present invention may be utilised to create cell lines.

Accordingly, another aspect of the present invention is directed to a method of modulating one or more endothelial cell functional characteristics in a mammal, said method comprising modulating the functional level of sphingosine kinase wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell characteristics.

More particularly, said method is directed to modulating one or more vascular endothelial cell functional characteristics in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cell relative to normal endothelial cell characteristics.

Still more particularly, said vascular endothelial cell is a CD34+ endothelial cell.

In one preferred embodiment, there is provide a method of modulating vascular endothelial cell proliferation in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level enhances the proliferation of said endothelial cell relative to normal
5 endothelial cell proliferation.

In another preferred embodiment, there is provided the method of modulating vascular endothelial cell viability in a mammal, said method comprising modulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said
10 sphingosine kinase level enhances the viability of said vascular endothelial cell relative to normal endothelial cell viability.

In yet another preferred embodiment, there is provided a method of modulating the CD34⁺ endothelial cell progenitor phenotype in a mammal, said method comprising modulating
15 the functional level of said sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level maintains the CD34⁺ endothelial cell progenitor phenotype.

In accordance with these preferred embodiments, most preferably modulation is up
20 regulation of the subject functional characteristics.

A further aspect of the present invention relates to the use of the invention in relation to the treatment and/or prophylaxis of disease conditions or other unwanted conditions. Without
25 limiting the present invention to any one theory or mode of action, the development of methodology which facilitates enhancement of endothelial cell proliferation, viability and the maintenance of the progenitor CD34⁺ endothelial cell phenotype provides a means of rapidly and efficiently expanding endothelial cell populations either *in vitro* or *in vivo*.

The fact that the viability of these cells can be enhanced renders the invention particularly useful in situations where ideal environmental factors may not be present. In this regard,
30 the inventors have developed herewith a means of generating particularly robust populations of endothelial cells. In particularly preferred embodiments, the method of the

present invention may be utilised to establish vascular grafts, to induce or seed vascularisation of tissue or organ grafts or to induce vascularisation of de-vascularised regions such as regions of amyloid plaque deposition. In another example the method of the present invention could be utilised to deliver drugs to the vascular system via
5 endothelial cells which may require the phenotypic features induced by sphingosine kinase overexpression in order to provide the desired survival or maturation conditions. Further, maintaining populations of immature endothelial cells may be useful to the extent that such cells are required in order to facilitate their stimulation and differentiation along a particular cell lineage, even a non-vascular cell lineage such as the differentiation to
10 muscle cells. Sphingosine kinase overexpression would be useful in this context since populations of immature proliferating endothelial cells could be maintained in a effective manner.

The present invention therefore contemplates a method for the treatment and/or
15 prophylaxis of a condition characterised by inadequate endothelial cell functioning in a mammal, said method comprising up-regulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level up-regulates one or more functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

20 Reference to "inadequate functioning" should be understood as a reference to under active endothelial cell functioning, to physiologically normal functioning which is inappropriate in that it is too low or to the absence of functioning. In this regard, reference to "functioning" should be understood as a reference to any one or more of the normal
25 functional characteristics as hereinbefore defined. Reference to "inadequate functioning" should also be understood to include reference to the presence of insufficient numbers of progenitor cells to differentiate along the endothelial cell pathway. For example, in certain situations, such as wound healing and tissue/organ transplantation, there may be very low levels of CD34⁺ progenitor cells available to differentiate along the endothelial cell
30 pathway. The method of the present invention provides a means of not only generating endothelial cell progenitor expansion, but also means of maintaining a population of these

More particularly, the present invention provides the method for the treatment and/or prophylaxis of a condition characterised by inadequate vascular endothelial cell functioning in a mammal, said method comprising up-regulating the functional level of sphingosine kinase in said mammal wherein inducing over-expression of said sphingosine kinase level up-regulates one or more functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

Preferably said condition is vascular engraftment, wound repair, tissue/organ transplantation or the repair of devascularised tissue.

In a most preferred embodiment, said up-regulated functional characteristic is one or more of enhanced endothelial cell proliferation, enhanced endothelial cell viability and/or maintenance of the CD34⁺ endothelial cell progenitor phenotype.

In a most preferred embodiment, there is provided the method for the treatment and/or prophylaxis of a condition characterised by inadequate vascular endothelial cell functioning in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce over-expression of a functional level of sphingosine kinase.

Reference to "agent" should be understood to have the same meaning as hereinbefore defined. However, in the context of this aspect of the present invention reference to "agent" should also be understood as a reference to a population of endothelial cells which have been treated in accordance with the method of the present invention. For example, prophylactically or therapeutically treating a condition characterised by inadequate vascular endothelial cell functioning may be achieved by introducing to the patient a population of endothelial cells which exhibit one or more of the improved functional characteristics which are obtainable in accordance with the method of the present invention. For example, a population of suitably treated CD34⁺ endothelial cell progenitors may be introduced to a site which requires revascularisation such as a site of

wound repair or a site of abnormal devascularisation (such as would occur where amyloid plaques are deposited).

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of the particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

The present invention further contemplates a combination of therapies, such as the administration of the modulatory agent together with other proteinaceous or non-proteinaceous molecules which may facilitate the desired therapeutic or prophylactic outcome.

Administration of molecules of the present invention hereinbefore described [herein collectively referred to as "modulatory agent"], in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends,

for example, on the human or animal and the modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day.

Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraocularly, intrathecally, intracerebrally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant. Preferably, said route of administration is oral.

In accordance with these methods, the agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. For example, the subject sphingosine kinase may be administered

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together with an agonistic agent in order to enhance its effects. Alternatively, in the case of organ tissue transplantation, the sphingosine kinase may be administered together with immunosuppressive drugs. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of
5 molecules. These molecules may be administered in any order.

Another aspect of the present invention relates to the use of an agent capable of modulating the functional level of sphingosine kinase in the manufacture of a medicament for the modulation of one or more endothelial cell functional characteristics in a mammal wherein
10 inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cells relative to normal endothelial cell functional characteristics.

In another aspect, the present invention relates to the use of sphingosine kinase or a nucleic acid encoding sphingosine kinase in the manufacture of a medicament for the modulation of one or more endothelial cell functional characteristics in a mammal wherein inducing over-expression of said sphingosine kinase level modulates one or more of the functional characteristics of said endothelial cells relative to normal endothelial cell functional
15 characteristics.

20 According to these preferred embodiments, the subject endothelial cells are preferably vascular endothelial cells and even more preferably, CD34+ vascular endothelial cells.

The term "mammal" and "subject" as used herein includes humans, primates, livestock animals (eg. sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer). Preferably, the mammal is human or a laboratory test animal. Even more preferably, the mammal is a human.

30 In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined and one or more

pharmaceutically acceptable carriers and/or diluents. Said agents are referred to as the active ingredients

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions
5 (where water soluble) or dispersions and sterile powders for the extemporaneous
preparation of sterile injectable solutions or dispersion or may be in the form of a cream or
other form suitable for topical application. It must be stable under the conditions of
manufacture and storage and must be preserved against the contaminating action of
microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion
10 medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene
glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and
vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating
such as lecithin, by the maintenance of the required particle size in the case of dispersion
and by the use of surfactants. The preventions of the action of microorganisms can be
15 brought about by various antibacterial and antifungal agents, for example, parabens,
chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be
preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged
absorption of the injectable compositions can be brought about by the use in the
compositions of agents delaying absorption, for example, aluminum monostearate and
20 gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the
required amount in the appropriate solvent with various of the other ingredients
enumerated above, as required, followed by filtered sterilisation. Generally, dispersions
25 are prepared by incorporating the various sterilised active ingredient into a sterile vehicle
which contains the basic dispersion medium and the required other ingredients from those
enumerated above. In the case of sterile powders for the preparation of sterile injectable
solutions, the preferred methods of preparation are vacuum drying and the freeze-drying
technique which yield a powder of the active ingredient plus any additional desired
30 ingredient from previously sterile-filtered solution thereof.

- When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the
- 5 active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The
- 10 amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μ g and 2000 mg of active compound.
- 15 The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of
- 20 wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and
- 25 propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.
- 30 The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule

encoding sphingosine kinase or a modulatory agent as hereinbefore defined. The vector may, for example, be a viral vector. The pharmaceutical composition may also comprise endothelial cell populations which have been treated in accordance with the method of the present invention.

5

Still another aspect of the present invention is directed to a method of generating an endothelial cell, which endothelial cell is characterised by the modulation of one or more functional characteristics relative to normal endothelial cell functional characteristics, said method comprising inducing over-expression of the functional level of sphingosine kinase

10

in said cell.

Yet another aspect of the present invention is directed to the endothelial cells which are generated in accordance with the methods defined herein.

15

Still yet another aspect of the present invention is directed to the use of endothelial cells developed in accordance with the method defined herein in the treatment and/or prophylaxis of conditions characterised by inadequate endothelial cell functioning.

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MEDVET SCIENCE PTY LTD

by its Patent Attorneys

DAVIES COLLISON CAVE

Figure 1

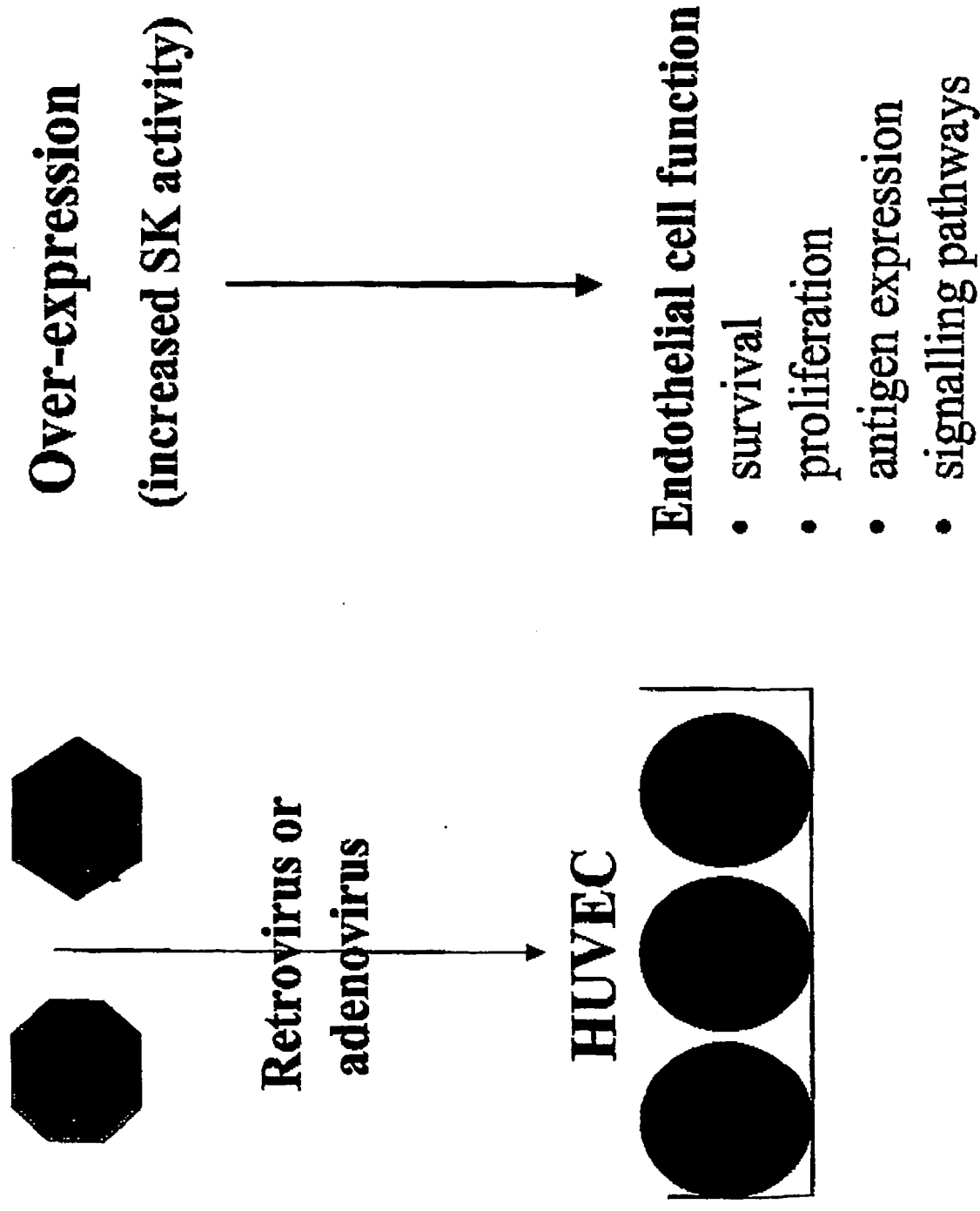


Figure 2

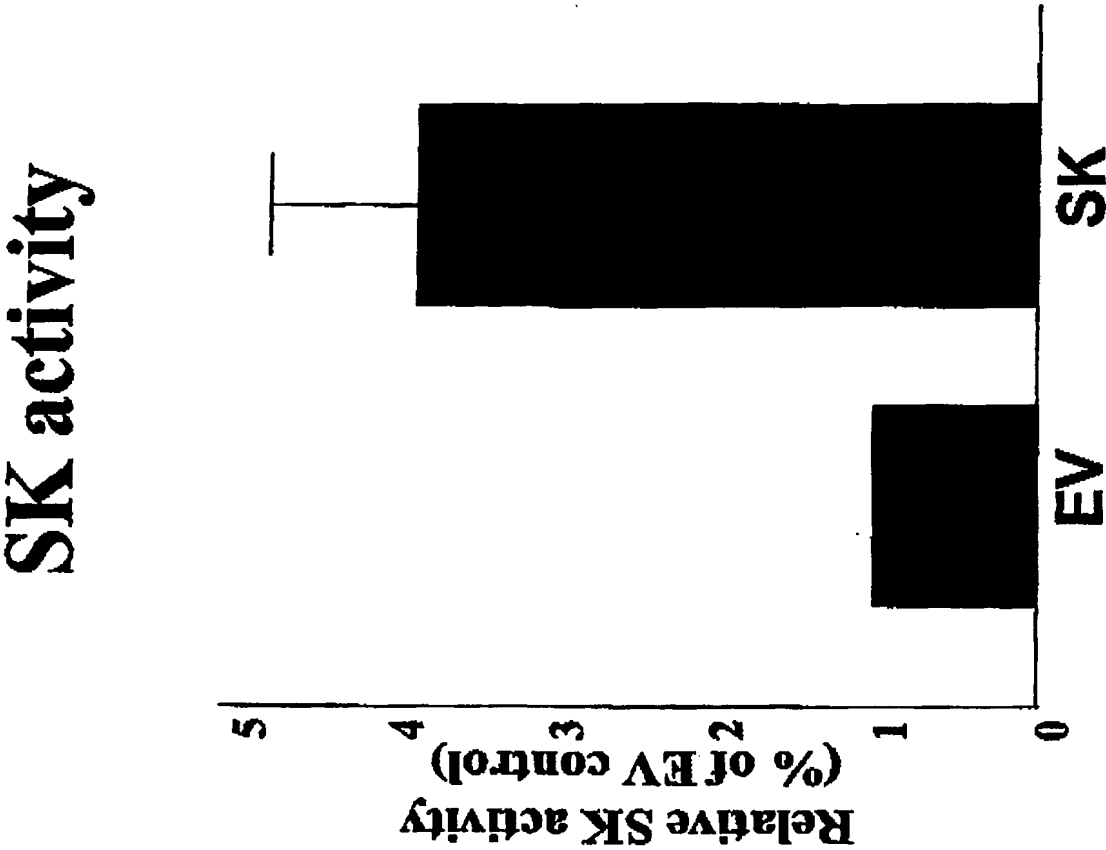
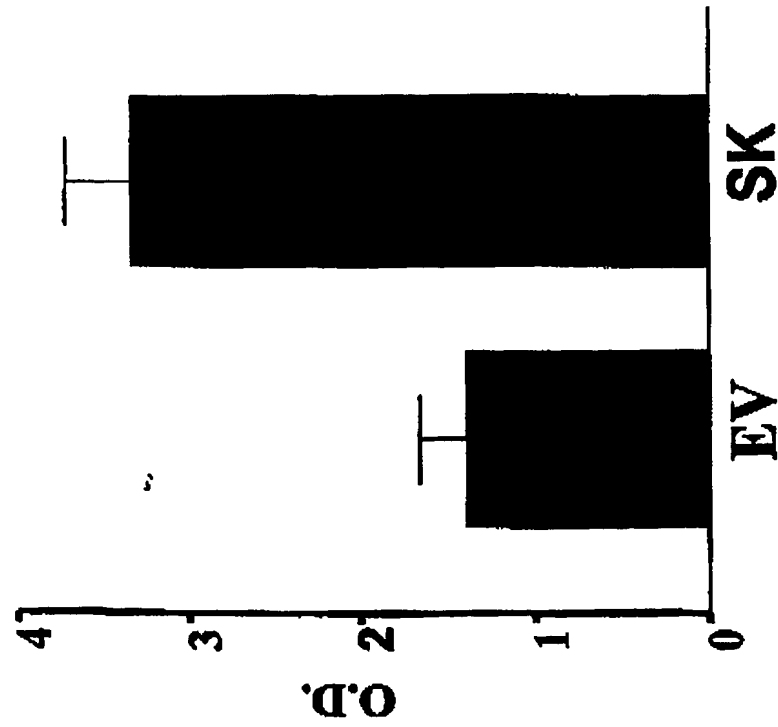


Figure 3

Proliferation



Survival

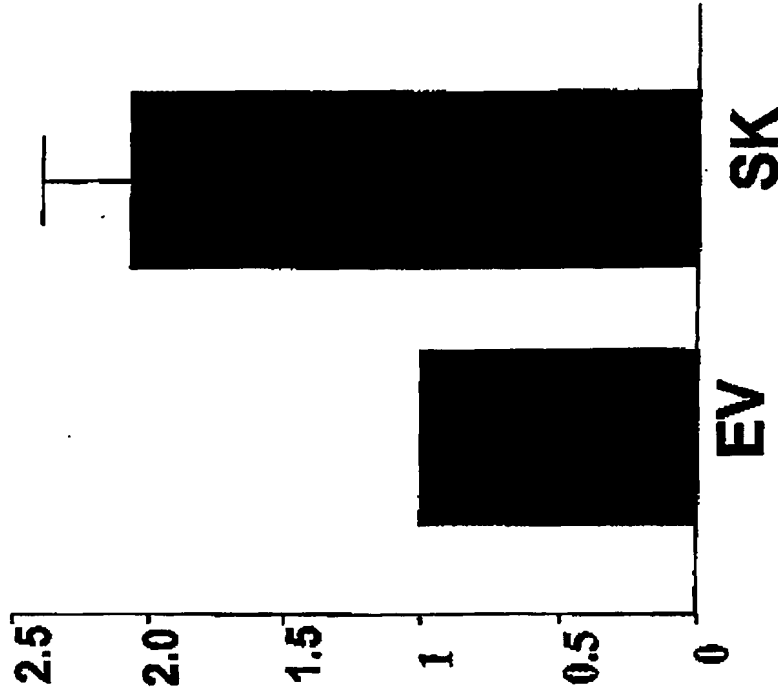


Figure 4

Overexpression of SK Results in Resistance to Apoptosis

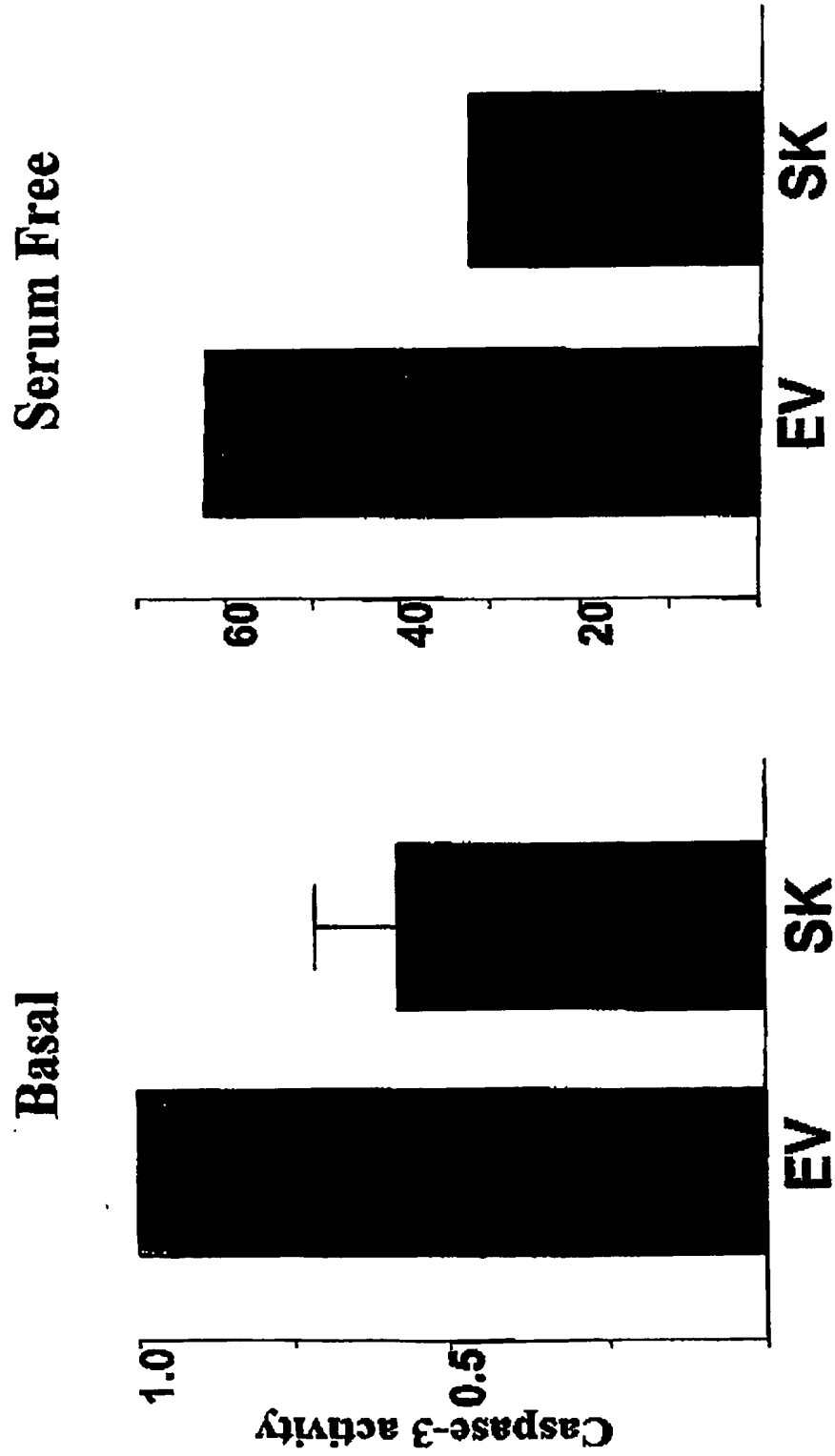


Figure 5

The PI3K Pathway is Involved in Mediating SK- Induced Survival

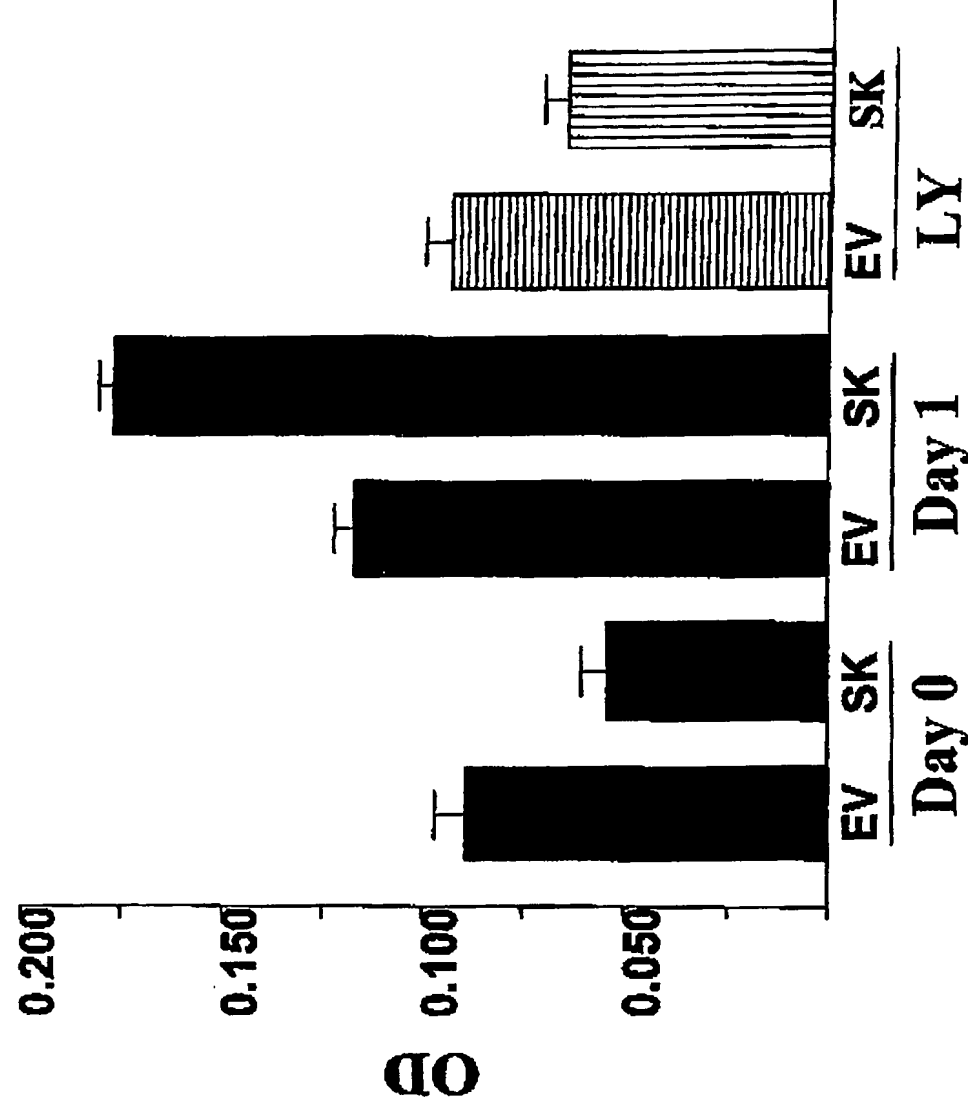


Figure 6

**Over-expression of SK promotes cell survival
in EC grown in suspension cultures**

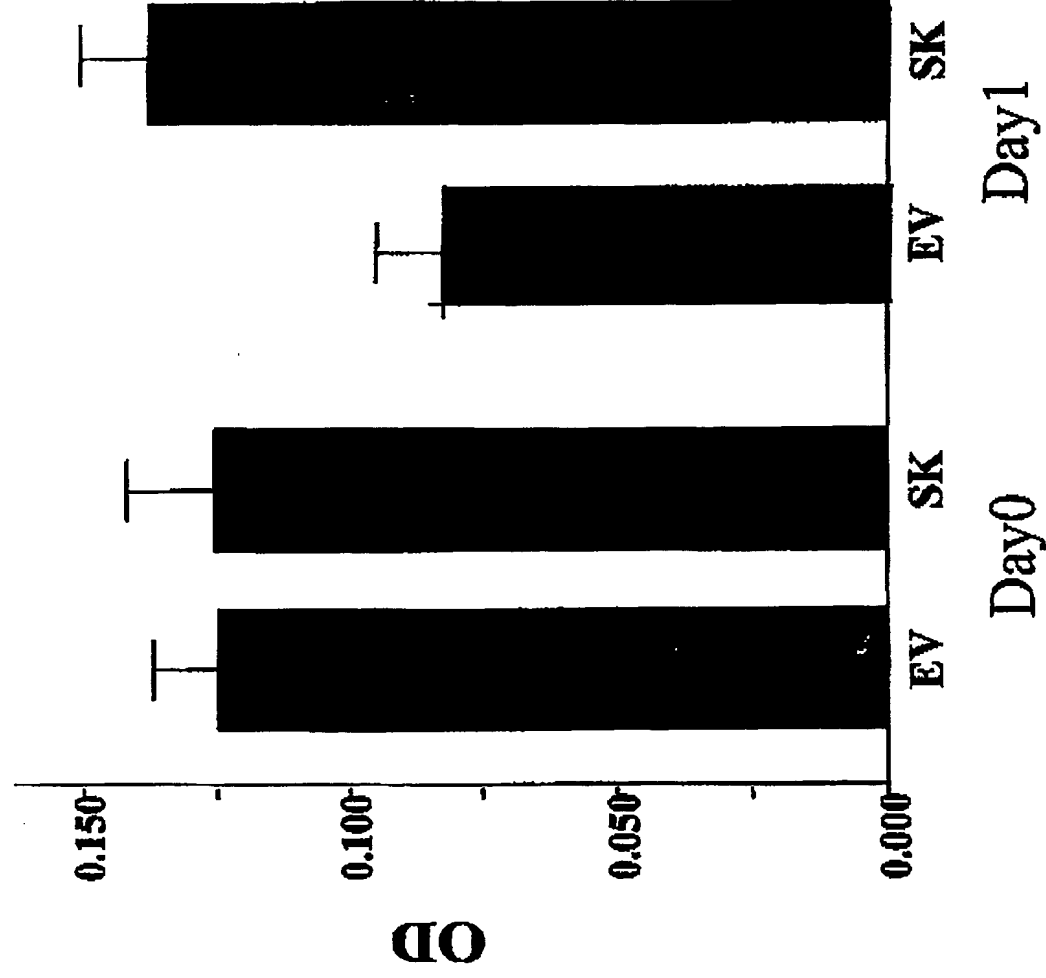


Figure 7

Over-expression of SK maintains CD34 expression in EC

